

FINAL REPORT

Test Facility Study No. 514868

Evaluation of the Eye Hazard Potential of MLA-3202 using the Bovine Corneal Opacity and Permeability Test (BCOP Test)

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26 August 2016

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1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

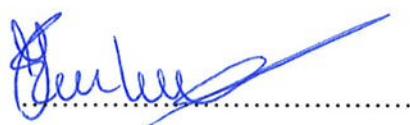
All phases of this study performed by the test facility were conducted in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals, 26 November 1997 (C(97) 186 Final);
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature:



Name: I.M.J. Burlings, PhD.

Title: Study Director

Date: 26 August 2016

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands.

Study title: Evaluation of the eye hazard potential of MLA-3202 using the bovine corneal opacity and permeability test (BCOP test).

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project 514868

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Study Plan Report	12-Jul-2016 17-Aug-2016	12-Jul-2016 17-Aug-2016	12-Jul-2016 17-Aug-2016
Process	Test Substance Receipt Test Substance Handling	09-May-2016	20-May-2016	24-May-2016
	Genetic and In Vitro Toxicology Test Substance Handling Exposure Observations/Measurements Specimen Handling	13-Jun-2016	30-Jun-2016	04-Jul-2016

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature: 

Name: C. Mitchell B.Sc., FRQA
Head of Quality Assurance

Date: 24. Aug. 2016

3. SUMMARY

Evaluation of the eye hazard potential of MLA-3202 using the Bovine Corneal Opacity and Permeability test (BCOP test).

This report describes the potency of chemicals to induce serious eye damage using isolated bovine corneas. The eye damage of MLA-3202 was tested through topical application for 10 minutes.

The study procedures described in this report were based on the most recent OECD guideline.

Batch RC-1045 of MLA-3202 was a clear amber-red liquid. The test item was applied as it is (750 µl) directly on top of the corneas.

The negative control responses for opacity and permeability were less than the upper limits of the laboratory historical range indicating that the negative control did not induce irritancy on the corneas. The mean *in vitro* irritancy score of the positive control (Ethanol) was 62 and was within two standard deviations of the current historical positive control mean. It was therefore concluded that the test conditions were adequate and that the test system functioned properly.

MLA-3202 did not induce ocular irritation through both endpoints, resulting in a mean *in vitro* irritancy score of 2.3 after 10 minutes of treatment.

Since MLA-3202 induced an IVIS ≤ 3, no classification is required for eye irritation or serious eye damage.

4. INTRODUCTION

4.1. Study schedule

Experimental starting date : 19 July 2016
Experimental completion date : 19 July 2016

4.2. Purpose

The aim of this study was to evaluate the eye hazard potential of MLA-3202 as measured by its ability to induce opacity and increase permeability in an isolated bovine cornea.

Background of the test system

The Bovine Corneal Opacity and Permeability Assay (BCOP) measures two important components which are predictive of irritation, corneal opacity and permeability.

The test consists of topical application of MLA-3202 on the epithelium of the bovine cornea for 10 minutes. MLA-3202 was applied undiluted. After exposure the cornea was thoroughly rinsed to remove the test item and incubated for 2 hours with fresh medium followed by opacity measurement and the permeability of the corneas was determined after a 90 minutes incubation period with sodium fluorescein.

4.3. Guidelines

The study procedures described in this report are in compliance with the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 437: " Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage"(adopted July 26, 2013).

4.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed. Responsible personnel

4.4.1. Test facility

Study Director I.M.J. Eurlings, PhD.

4.4.2. Sponsor Representative

Study Monitor Audrey Batoon, Ph.D.

5. MATERIALS AND METHODS

5.1. Test item

5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

5.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Stability at higher temperatures	Stable
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

5.2. Reference items

5.2.1. Negative control

A negative control, physiological saline (Eurovet Animal Health, Bladel, The Netherlands) was included to detect non-specific changes in the test system and to provide a baseline for the assay endpoints.

5.2.2. Positive control

Identification	Ethanol
Identification number	RS532
CAS Number	64-17-5
Molecular formula	C ₂ H ₅ OH
Molecular weight	46.07
Appearance	Clear colourless liquid
Batch	K47177483
Purity	≥99.9%
Storage conditions	At room temperature
Stable under storage conditions until	31 October 2020

5.3. Test item preparation

No correction was made for the purity/composition of the test item.

The test item was tested neat.

5.4. Test system

Test System:	Bovine eyes were used as soon as possible after slaughter.
Rationale:	In the interest of sound science and animal welfare, a sequential testing strategy is recommended to minimise the need of <i>in vivo</i> testing (1-6). As a consequence a validated and accepted <i>in vitro</i> test for eye irritation should be performed before <i>in vivo</i> tests are conducted. One of the proposed validated <i>in vitro</i> eye irritation tests is the Bovine Corneal Opacity and Permeability (BCOP) test.
Source:	Bovine eyes from young cattle were obtained from the slaughterhouse (Vitelco, 's Hertogenbosch, The Netherlands), where the eyes were excised by a slaughterhouse employee as soon as possible after slaughter.
Transport:	Eyes were collected and transported in physiological saline in a suitable container under cooled conditions.

5.5. Study design

5.5.1. Preparation of corneas

The eyes were checked for unacceptable defects, such as opacity, scratches, pigmentation and neovascularization by removing them from the physiological saline and holding them in the light. Those exhibiting defects were discarded.

The isolated corneas were stored in a petri dish with cMEM (Earle's Minimum Essential Medium (Life Technologies, Bleiswijk, The Netherlands) containing 1% (v/v) L-glutamine (Life Technologies) and 1% (v/v) Foetal Bovine Serum (Life Technologies)). The isolated corneas were mounted in a corneal holder (one cornea per holder) of BASF (Ludwigshafen, Germany) with the endothelial side against the O-ring of the posterior half of the holder. The anterior half of the holder was positioned on top of the cornea and tightened with screws. The compartments of the corneal holder were filled with cMEM of $32 \pm 1^\circ\text{C}$. The corneas were incubated for the minimum of 1 hour at $32 \pm 1^\circ\text{C}$.

5.5.2. Cornea selection and Opacity reading

After the incubation period, the medium was removed from both compartments and replaced with fresh cMEM. Opacity determinations were performed on each of the corneas using an opacitometer (BASF-OP3.0, BASF, Ludwigshafen, Germany). The opacity of each cornea was read against a cMEM filled chamber, and the initial opacity reading thus determined was recorded. Corneas that had an initial opacity reading higher than 7 were not used. Three corneas were selected at random for each treatment group.

5.5.3. Treatment of corneas and opacity measurements

The medium from the anterior compartment was removed and 750 µl of either the negative control, positive control (Ethanol) or test item was introduced onto the epithelium of the cornea. The holders were slightly rotated, with the corneas maintained in a horizontal position, to ensure uniform distribution of the control or the test item over the entire cornea. Corneas were incubated in a horizontal position for 10 ± 1 minutes at $32 \pm 1^\circ\text{C}$. After the incubation the solutions were removed and the epithelium was washed with MEM with phenol red (Earle's Minimum Essential Medium, Life Technologies) and thereafter with cMEM. Possible pH effects of the test item on the corneas were recorded. The medium in the posterior compartment was removed and both compartments were refilled with fresh cMEM.

Subsequently the corneas were incubated for 120 ± 10 minutes at $32 \pm 1^\circ\text{C}$. After the completion of the incubation period opacity determination was performed. Each cornea was inspected visually for dissimilar opacity patterns.

5.5.4. Opacity measurement

The opacity of a cornea was measured by the diminution of light passing through the cornea. The light was measured as illuminance (I = luminous flux per area, unit: lux) by a light meter.

The opacity value (measured with the device OP-KIT) was calculated according to:

$$\text{Opacity} = \frac{\frac{I_0}{I} - 0.9894}{0.0251}$$

With I_0 the empirically determined illuminance through a cornea holder but with windows and medium, and I the measured illuminance through a holder with cornea.

The change in opacity for each individual cornea (including the negative control) was calculated by subtracting the initial opacity reading from the final post-treatment reading. The corrected opacity for each treated cornea with the test item or positive control was calculated by subtracting the average change in opacity of the negative control corneas from the change in opacity of each test item or positive control treated cornea.

The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of the treated corneas for each treatment group.

5.5.5. Application of sodium fluorescein

Following the final opacity measurement, permeability of the cornea to Na-fluorescein (Sigma-Aldrich, Germany) was evaluated.

The medium of both compartments (anterior compartment first) was removed. The posterior compartment was refilled with fresh cMEM. The anterior compartment was filled with 1 ml of 4 mg Na-fluorescein (Sigma-Aldrich Chemie GmbH, Germany)/ml cMEM solution. The holders were slightly rotated, with the corneas maintained in a horizontal position, to ensure uniform distribution of the sodium-fluorescein solution over the entire cornea. Corneas were incubated in a horizontal position for 90 ± 5 minutes at $32 \pm 1^\circ\text{C}$.

5.5.6. Permeability determinations

After the incubation period, the medium in the posterior compartment of each holder was removed and placed into a sampling tube labelled according to holder number. $360 \mu\text{l}$ of the medium from each sampling tube was transferred to a 96-well plate. The optical density at 490 nm (OD_{490}) of each sampling tube was measured in triplicate using a microplate reader (TECAN Infinite® M200 Pro Plate Reader). Any OD_{490} that was 1.500 or higher was diluted to bring the OD_{490} into the acceptable range (linearity up to OD_{490} of 1.500 was verified before the start of the experiment). OD_{490} values of less than 1.500 were used in the permeability calculation.

The mean OD_{490} for each treatment was calculated using cMEM corrected OD_{490} values. If a dilution has been performed, the OD_{490} of each reading of the positive control and the test item was corrected for the mean negative control OD_{490} before the dilution factor was applied to the reading.

5.6. Interpretation

5.6.1. In vitro irritancy score

The mean opacity and mean permeability values (OD_{490}) were used for each treatment group to calculate an *in vitro* score:

In vitro irritancy score (IVIS) = mean opacity value + (15 x mean OD_{490} value)

Additionally the opacity and permeability values were evaluated independently to determine whether the test item induced irritation through only one of the two endpoints.

The IVIS cut-off values for identifying the test items as inducing serious eye damage (UN GHS Category 1) and test items not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter:

<i>In vitro</i> score range	UN GHS
≤ 3	No Category
$> 3; \leq 55$	No prediction can be made
> 55	Category 1

5.6.2. Acceptability of the assay

The assay is considered acceptable if:

- The positive control gives an *in vitro* irritancy score that falls within two standard deviations of the current historical mean.
- The negative control responses should result in opacity and permeability values that are less than the upper limits of the laboratory historical range.

5.7. List of deviations

5.7.1. List of study plan deviations

1. One of the negative control eyes was excluded since it was slightly translucent resulting in an opacity value which was outside the normal range.

Evaluation: Since the other two eyes met the criteria and the test item results were not influenced by this exclusion, this does not affect the study outcome.

The study integrity was not adversely affected by the deviation.

5.7.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- Magellan Tracker 7.0 (TECAN, Austria) for optical density measurement.

7. RESULTS

MLA-3202 was tested neat.

[Table 1 \(APPENDIX 1\)](#) summarizes the opacity, permeability and *in vitro* irritancy scores of MLA-3202 and the controls. The opacity, permeability and *in vitro* scores of the individual corneas are shown in [Table 2-5](#).

The individual *in vitro* irritancy scores for the negative controls ranged from -1.3 to -0.4 (study plan deviation 1). The individual positive control *in vitro* irritancy scores ranged from 53 to 73 for Ethanol ([APPENDIX 2, Table 5](#)). The corneas treated with the positive control item were turbid after the 10 minutes of treatment.

The corneas treated with MLA-3202 showed opacity values ranging from 1.3 to 2.7 and permeability values ranging from -0.006 to 0.027. The corneas were translucent after the 10 minutes of treatment with MLA-3202. No pH effect of the test item was observed on the rinsing medium. Hence, the *in vitro* irritancy scores ranged from 1.2 to 3.1 after 10 minutes of treatment with MLA-3202.

8. DISCUSSION AND CONCLUSION

The negative control responses for opacity and permeability were less than the upper limits of the laboratory historical range indicating that the negative control did not induce irritancy on the corneas. The mean *in vitro* irritancy score of the positive control (Ethanol) was 62 and was within two standard deviations of the current historical positive control mean.

([APPENDIX 3, Table 6](#)). It was therefore concluded that the test conditions were adequate and that the test system functioned properly.

MLA-3202 did not induce ocular irritation through both endpoints, resulting in a mean *in vitro* irritancy score of 2.3 after 10 minutes of treatment.

Since MLA-3202 induced an IVIS ≤ 3 , no classification is required for eye irritation or serious eye damage.

9. REFERENCES

1. Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No. 405, "Acute Eye Irritation / Corrosion", Paris Cedex, 2002.
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7. European Community (EC). Commission regulation (EC) No. 440/2008, Part B: Methods for the Determination of Toxicity and other health effects, Guideline B.47 "Bovine corneal opacity and permeability method for identifying ocular corrosives and severe irritants ". Official Journal of the European Union No. L324; Amended by EC No. 1152/2010 No. L142, 09 December 2010.
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APPENDIX 1
Summary Table
Table 1
Summary of opacity, permeability and *in vitro* scores

Treatment	Mean Opacity ¹	Mean Permeability ¹	Mean <i>In vitro</i> Irritation Score ^{1, 2}
Negative control	-1.0	0.010	-0.8
Positive control (Ethanol)	22.3	2.636	61.8
MLA-3202	2.2	0.006	2.3

¹ Calculated using the negative control mean opacity and mean permeability values for the positive control and test item.

² *In vitro* irritancy score (IVIS) = mean opacity value + (15 x mean OD₄₉₀ value).

APPENDIX 2
Individual opacity, permeability and in vitro scores
Table 2
Opacity score

Treatment	Opacity before treatment	Opacity after treatment	Final Opacity ¹	Negative control corrected Final Opacity ²	Mean Final Opacity
Negative control	4.0	2.6	-1.4		-1.0
	2.7	2.1	-0.6		
	1.9 ⁵	7.3 ⁵	5.5 ⁵		
Positive control	3.0	21.3	18.4	18.4	22.3
	1.6	23.8	22.2	22.2	
	2.2	28.4	26.2	26.2	
MLA-3202	3.5	6.2	2.7	2.7	2.2
	3.0	4.3	1.3	1.3	
	1.7	4.3	2.6	2.6	

¹ Final Opacity = Opacity after treatment – Opacity before treatment.

² Negative control corrected Final Opacity = Final opacity – Mean final opacity negative control.³

³ In case the mean final opacity of the negative control is below zero, no correction will be made.

⁴ Calculations are made without rounding off.

⁵ Eye excluded from analysis (study plan deviation 1)

Table 3
Permeability score individual values (uncorrected)

Treatment	Dilution factor	OD ₄₉₀ 1	OD ₄₉₀ 2	OD ₄₉₀ 3	Average OD	Final OD	Mean final negative control
Negative control	1	0.004	0.005	0.005	0.005	0.005	0.010
	1	0.020	0.014	0.010	0.015	0.015	
	1	0.001 ²	-0.001 ²	0.001 ²	0.000 ²	0.000 ²	
Positive control	6	0.398	0.400	0.399	0.399	2.394	
	6	0.572	0.575	0.569	0.572	3.432	
	6	0.377	0.374	0.377	0.376	2.256	
MLA-3202	1	0.037	0.037	0.037	0.037	0.037	
	1	0.001	0.005	0.004	0.003	0.003	
	1	0.007	0.007	0.010	0.008	0.008	

¹ Calculations are made without rounding off.

² Eye excluded from analysis (study plan deviation 1)

APPENDIX 2 - continued -
Individual opacity, permeability and in vitro scores

Table 4
Permeability score individual values (corrected)

Treatment	Dilution factor	Negative control corrected OD ₄₉₀ 1 ¹	Negative control corrected OD ₄₉₀ 2 ¹	Negative control corrected OD ₄₉₀ 3 ¹	Negative control corrected OD ₄₉₀ Average	Negative control corrected final OD ₄₉₀	Average OD
Positive control	6	0.388	0.390	0.389	0.389	2.336	2.636
	6	0.562	0.565	0.559	0.562	3.374	
	6	0.367	0.364	0.367	0.366	2.198	
MLA-3202	1	0.027	0.027	0.027	0.027	0.027	0.006
	1	-0.009	-0.005	-0.006	-0.006	-0.006	
	1	-0.003	-0.003	0.000	-0.002	-0.002	

¹ OD₄₉₀ values corrected for the mean final negative control permeability (0.010).

² Calculations are made without rounding off.

Table 5
In Vitro irritancy score

Treatment	Final Opacity ²	Final OD ₄₉₀ ²	In vitro Irritancy Score ¹
Negative control	-1.4	0.005	-1.3
	-0.6	0.015	-0.4
	5.5 ³	0.000 ³	5.5 ³
Positive control	18.4	2.336	53.4
	22.2	3.374	72.8
	26.2	2.198	59.2
MLA-3202	2.7	0.027	3.1
	1.3	-0.006	1.2
	2.6	-0.002	2.6

¹ In vitro irritancy score (IVIS) = opacity value + (15 x OD₄₉₀ value).

² Positive control and test item are corrected for the negative control.

³ Eye excluded from analysis (study plan deviation 1)

APPENDIX 3
Historical control data
Table 6
Historical control data for the BCOP studies

	Negative control			Positive control
	Opacity	Permeability	<i>In vitro</i> Irritancy Score	<i>In vitro</i> Irritancy Score
Range	-2.9 – 3.0	-0.016 – 0.029	-2.8 – 3.0	35.8 – 72.9
Mean	0.25	0.00	0.34	56.58
SD	1.07	0.01	1.18	12.51
n	51	43	45	21

SD = Standard deviation

n = Number of observations

The above mentioned historical control data range of the controls were obtained by collecting all data over the period of February 2015 to May 2016.

APPENDIX 4

Certificate of analysis



Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis
Analytical REACH Scientist, Analytical Services

3/7/16

Blake Lewis
Analytical REACH Scientist, Analytical Services

Date _____

Blair Lewis
Analytical REACH Scientist, Analytical

John M. Moore.

Son A5N
Albert J. Nitowski
S. T. 10-1-11